CHAPTER 1.5 - WET Data Reporting, Review, and Interpretation

This chapter describes the WET test review process generally followed by the Biomonitoring Coordinator. This chapter is provided for staff, permittees, and others who want to understand why review decisions were made; it also discusses common data patterns and provides guidance on dose-response, confidence intervals, and other information.

NOTICE: This document is intended solely as guidance, and does not contain any mandatory requirements except where requirements found in statute or administrative rule are referenced. This guidance does not establish or affect legal rights or obligations, and is not finally determinative of any of the issues addressed. This guidance does not create any rights enforceable by any party in litigation with the State of Wisconsin or the Department of Natural Resources. Any regulatory decisions made by the Department of Natural Resources in any matter addressed by this guidance will be made by applying the governing statutes and administrative rules to the relevant facts.

"Whole Effluent Toxicity (WET) Test Report Forms"

According to the "*State of Wisconsin Aquatic Life Toxicity Testing Methods Manual, 2nd edition*" (Methods Manual), WET Test Report Forms must be submitted for demonstrating test completion and compliance with a WPDES permit. The WET Test Report Form and instructions for completing the form can be found in Section 6 of the Methods Manual. Forms and attachments must contain all information needed to determine compliance with WET requirements, as specified in the WPDES permit and Methods Manual. Reports must contain a description and justification of any abnormal procedures, conditions, or manipulations used in the test(s). The permittee and lab should also provide any attachments or additional information which they believe to be relevant to the test. All other test documentation (e.g. bench sheets, record books, etc.) needed to fulfill manual or QA requirements must be maintained at the lab for laboratory certification purposes (see Section 3.16, of the Methods Manual).

WET Test Report Forms must include observations made by the permittee or lab that may influence test results or data interpretation, such as: 1) unusual conditions (e.g., plant upsets, slug loads, weather conditions, etc.) during sampling periods, 2) deviations from test specifications or any sample manipulation (aeration, filtration, addition of chemicals, etc.) that is determined to be necessary for successful completion of a test, and/or 3) unusual behavior or appearance of test organisms (e.g., young developed in the brood pouch of the adults, but not released during the exposure period; partially or fully developed young released, but all dead at the end of the 24-h period; lethargy, hyperactivity, spots or filaments, discoloration, excessive ventilation, etc.).

Mailing and Routing Procedures for the WET Test Report Form

The Methods Manual requires that the original, complete, signed version of the WET Test Report Form be sent to the Biomonitoring Coordinator (Department of Natural Resources, WT/3, 101 South Webster St., P.O. Box 7921, Madison, WI 53707-7921) by the date specified in the WPDES permit. Upon receipt at the central office, forms are date-stamped so others can determine when they were received. Report forms are reviewed by the Biomonitoring Coordinator as soon as possible (usually within 1-3 weeks, depending on workload). The Biomonitoring Coordinator sends copies of the reviewed form to Basin Engineers (and sometimes Permit Coordinators) for tracking with permit conditions. If qualified staff are not available in the central office to review WET reports, administrative staff should be informed so that reports can be date stamped and immediately sent to Regional staff upon receipt.

Quality Assurance And Data Review And Reporting Process

It is widely accepted that WET testing methods are scientifically sound with respect to the data generated for various biological endpoints such as survival, growth, and reproduction. However, in order to insure continued program success, these methods must be conducted and interpreted by experienced WET professionals.

The Biomonitoring Coordinator is primarily responsible for the review and interpretation of WET test results, including the application of best professional judgment in the recognition and investigation of unusual test results. The Coordinator also provides technical advice and support to Department staff, permittees, and laboratories who must make decisions about WET requirements in WPDES permits. The Coordinator works closely with permittees and laboratories to evaluate and interpret WET test and toxicity reduction evaluation (TRE) information, especially in cases where effluent toxicity has been encountered. A thorough WET data review process includes many steps, including those described below.

Review and confirmation of test conditions - The Methods Manual requires certain conditions (e.g., temperature, number of replicates, chamber size and volume, lighting, etc.) that must be followed in all tests submitted for WPDES compliance. Before submitting results to the Department, the results of each test should be reviewed by permittees and lab staff to ensure that conditions were met within the flexibility provided by the Methods Manual. Labs should verify daily measurements to ensure that values are within the acceptable ranges allowed by the test methods and report whether these conditions were met on page 1 of the WET Test Report Form. Any deviations from Methods Manual requirements should be clearly reported on the report form. The Biomonitoring Coordinator will check and attempt to verify this information while reviewing WET test data.

Review of reference toxicant testing – Reference toxicant tests are conducted under the same conditions (e.g., test duration, conditions, endpoint) as effluent tests, substituting a known toxicant for effluent samples (s. 3.15 Methods Manual). Reference toxicant testing is an important quality control practice that is required in order to 1) determine the sensitivity of the test organisms over time, and 2) assess the quality and comparability of within- and between-laboratory test results. Reference toxicant test results can be used to help identify potential sources of variability, such as test organism health, difference among batches of organisms, changes in laboratory water or food quality, and performance by laboratory technicians. By standardizing reference toxicants, test results can be compared within the same laboratory and between different laboratories. (See Chapter 2.1 for guidance on how to evaluate reference toxicant data.) Labs must indicate on page 1 of the report form whether reference toxicant tests that were performed at the time of effluent testing were within acceptable limits.

Reference toxicant testing is usually required monthly for each test method routinely conducted in a laboratory. A "control chart" must be maintained for each combination of species and test condition. A control chart is a running plot of the 20 most recent test endpoints (LC_{50} or IC_{25}). These charts are used to evaluate the cumulative trend of these endpoints, which are examined to insure that they are within prescribed limits.

The Methods Manual requires that labs report reference toxicant results on a quarterly basis. The Biomonitoring Coordinator regularly evaluates the test results and control charts submitted by each laboratory. Data are reviewed to look for outliers (values falling outside the upper and lower control limits) and trends of increasing or decreasing sensitivity. If it is determined that a series of reference toxicant tests are out of an acceptable range, effluent testing conducted during the same period may be rejected. Lab performance is expected to improve with experience and control limits generated by experienced, quality labs should gradually narrow over time.

Review of Water Chemistry Data - Receiving water and effluent data for hardness, alkalinity, pH, total ammonia, and total residual chlorine are reported on WET Test Report Forms so that general sample characteristics can be assessed to determine their potential impact on test results. Values reported for hardness, alkalinity, and ammonia are from measurements taken upon arrival at the lab. Values reported for chlorine and pH are from measurements taken after samples have been warmed to test temperatures and just prior to use in tests.

Hardness, alkalinity, and ammonia data can be extremely important for determining whether test or effluent conditions have impacted test results. Test organisms may experience reduced survival and/or reproduction if exposed to waters that have hardness or alkalinity values significantly different from culture waters. Ammonia and pH values should be reviewed to determine whether ammonia may have caused toxicity. (In general, ammonia levels > 10 mg/l are of concern, if pH is above 8.0; at lower pHs, more ammonia is needed to cause toxicity.)

Review of test acceptability criteria - The Methods Manual provides criteria that must be met in order for WET tests to be acceptable (e.g., minimum survival, reproduction, growth, variability, etc.). These criteria are set in order to insure that any effects noted during WET tests are due to the effluent being tested, and not due to dilution water, lab error, or other factors. The WET Test Report Form provides spaces where it must be reported whether these criteria were met during each test. Tests not meeting the minimum test acceptability criteria may be considered invalid and need to be repeated (see Section 3.8 and 3.9 of the Methods Manual for details).

Review of test variability - The variability of each WET test, measured as coefficient of variation (%CV), must be reported with all tests. It is also recommended that laboratories maintain control charts for test variability. These control charts allow laboratories to assess individual test variability in the context of typical variability within the laboratory. High test variability can result in insensitive tests or unexpected dose-response relationships. Consult Chapter 2.9 & USEPA (2000) for additional guidance on WET test method variability.

Review of test results and dose-response relationships – WET test results must be presented tabularly and graphically on WET Test Report Forms. Replicate and mean survival, growth, and reproduction data are plotted against test concentrations in graphs found on pages 2 and 3 of the form. Graphs give a visual picture of the dose-response, variability of the data, and highlight suspicious data and outliers.

Section 5.3.3 of the Methods Manual requires dose-response relationships be reviewed to ensure that results are interpreted correctly. Based on review of a dose-response curve several determinations may be made, including: 1) dose-responses are reliable and should be used for determining compliance, 2) dose-responses are anomalous and should be explained, or 3) results are inconclusive and tests should be repeated within 30 days.

It should be noted that the determination of a valid dose-response relationship is not always clear cut. Permittees and labs should review dose-response information, highlight any potential problems on WET Test Report Forms, and discuss any abnormalities with the Biomonitoring Coordinator. Final decisions regarding the acceptability of tests based on dose-response information shall be made by the Biomonitoring Coordinator, as required by Section 5.3.3 of the Methods Manual.

How Is The Dose-Response Used When Interpreting WET Data?

The dose-response is a fundamental concept of toxicology. This concept assumes that there is a relationship between the dose (or concentration) of a toxicant and the measured response. This relationship is the basis for the determination of point estimates (LC_{50}, IC_{25}) in WET testing. A biological response (e.g., mortality) is measured at a range of concentrations to develop a dose-response curve. In general, more severe responses are expected at higher concentrations and less severe responses at lower concentrations. (See Figure 1.5.1)

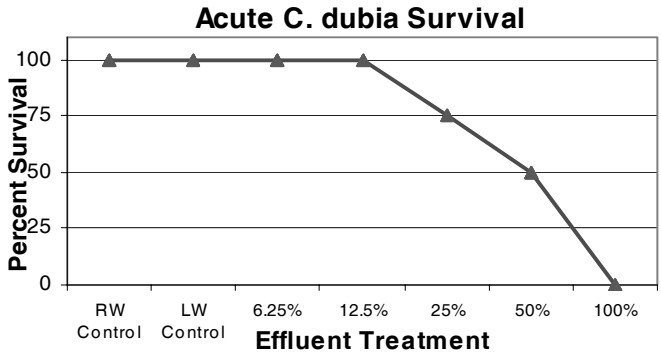

Figure 1.5.1. Example "normal" dose-response when toxicity is present.

Guidance is given here which may be used when evaluating the dose-response as a part of the data review and reporting process. Provided are examples of common patterns in WET test data, possible causes and solutions for unexpected patterns, and guidance on when data may be accepted or rejected based on the dose-response (or lack thereof). It should be noted that the determination of a valid dose-response relationship is not always clear cut. Data from some tests may suggest consultation with a professional toxicologist such as the Department's Biomonitoring Coordinator or professional lab staff. Tests that exhibit unexpected dose-response relationships may indicate a need for further investigation and possible retesting. In general, when unexpected or apparently anomalous dose-response relationships are encountered, the following is recommended:

- **Attempt to determine a cause for the response** - Test review and specific guidance may assist in determining a cause for an unexpected dose-response. Unexpected responses could be valid patterns or anomalies resulting from Type I test error (e.g., lab error, sampling problems, etc.), high test variability, or other causes. If a given effluent consistently produces an "unexpected" dose-response, there is likely a physical, chemical or biological cause. In situations where difficult-to-interpret dose-response relationships are produced consistently by a given effluent, consultation with a professional toxicologists is recommended.

- **Follow guidance for specific dose-response patterns** - Detailed guidance is given below which describes dose-response curves and provides examples of patterns that may be exhibited by WET test data. This section provides guidance in interpreting each dose-response pattern using a step-by-step review process. Based on this review, the guidance may recommend acceptance of the calculated results as valid and reliable, explanation of the calculated results as anomalous, or retesting.

- **Coordination between the DNR, permittee, and laboratory** - It is often wise for the DNR, permittee, and laboratory personnel to work together to resolve difficult-to-interpret WET test data. Discussions should be initiated as soon as possible when questions arise regarding WET test results.

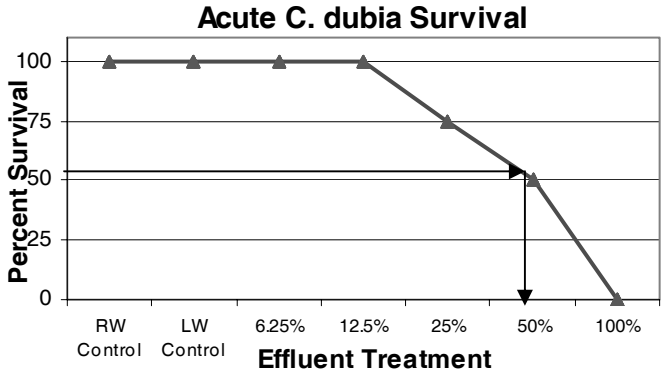

Figure 1.5.2. From the dose-response curve, a "point estimate effect concentration" (LC_{50}) can be calculated. In this example, LC_{50} = 50% effluent.

The dose-response concept is the basis for the determination of point estimates in WET testing. A biological response (mortality, growth, etc.) is measured at a range of effluent concentrations to develop a dose-response curve. From the resulting curve, a "point estimate effect concentration" (e.g., LC_{50}, IC_{25}) can be calculated (see Figure 1.5.2). The effect concentration is an estimate of the concentration of effluent that will produce a specific level of response (e.g., 50% mortality, 25% inhibition).

One assumption of this concept is that every toxicant should exhibit a dose-response relationship, assuming the appropriate response is measured and the concentration range evaluated is appropriate. Use of this concept can be helpful in determining whether an effluent possesses toxicity and in identifying anomalous test results. Tests that exhibit unexpected dose-response relationships may indicate a need for further testing. As noted above, if a given

effluent consistently produces an "unexpected" dose-response, there is likely a physical, chemical or biological cause which should be further investigated.

Common Dose-Response Relationships

Several dose-response patterns are described below using hypothetical test data. Each section provides guidance for interpreting each dose-response pattern. The focus is on determining a cause for unexpected patterns by recommending a step-by-step review process. After thorough review it may be determined that it is appropriate to accept the calculated endpoint (LC_{50} or IC_{25}) as valid and reliable, the calculated results are anomalous, or that retesting is needed. Test results should be reported for all tests conducted, even if retesting is recommended.

This guidance on dose-response relationships is for informational purposes only and it is not intended to be used to recommend frequent disqualification or repetition of WET tests. Several conditions should be considered when using the guidance in this chapter. First, unexpected dose-response relationships should not occur with any regular frequency. Second, it is not recommended to reject only those tests in which toxicity is found at or below the concentration of concern. If screening is to be done for unexpected dose-response relationships, all tests should be screened in a similar manner. Third, all results should be reported to and reviewed by the Department, including those disqualified and repeated. In most cases, the Biomonitoring Coordinator will make final decisions about whether test results are to be rejected based on unusual dose-response patterns (Methods Manual, s. 5.3.3).

1) **Ideal dose-response relationship.** This response pattern (see Figure 1.5.1) shows a clear dose-response relationship, with multiple effluent concentrations identified as significantly different from the control. There is a monotonic decrease in response, meaning that the response steadily decreases for each higher effluent concentration. This pattern is indicative of a well designed test with appropriately chosen concentrations that bracket the effluent's range of toxicity. Under these circumstances, point estimation techniques (LC_{50}, IC_{25}) recommended in the Methods Manual should provide reliable results.

2) **All or nothing response/Significant effects only at highest concentration.** The "all or nothing" response pattern is very common in WET tests. This pattern (Figure 1.5.3) is characterized by a transition from no significant effect at one concentration to a complete effect at the next higher concentration. This response pattern is most often characterized by only the highest test concentration producing a significantly different response from the control. This pattern represents a valid dose-response relationship and point estimation techniques recommended in the Methods Manual should provide reliable results.

   When this pattern of response is shown, the concentrations used for testing should be re-evaluated for future tests, especially when the highest concentration is at or near the concentration of concern. The precision of future point estimates may be improved by closer spacing of effluent concentrations or the addition of intermediate concentrations. This approach should be used only if historical testing of the effluent indicates consistency and the effect concentration is not likely to fall below the adjusted test concentration series.

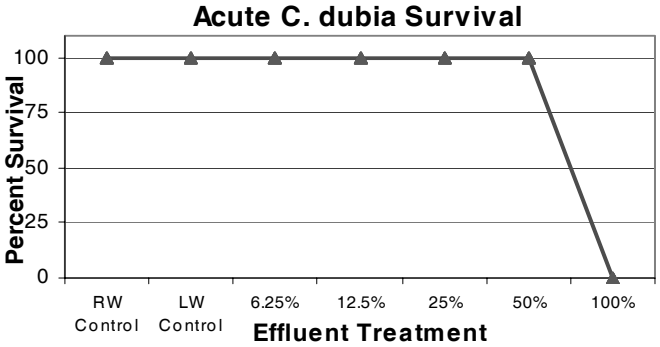

Figure 1.5.3 An example dose-response, showing an "all or nothing" response.

3) **Stimulatory response at low concentrations and detrimental effects at higher concentrations.** It is not uncommon for the lowest concentration in a toxicity test to demonstrate an effect that is greater than the control. The apparent stimulation of growth/reproduction by low toxicant doses is well known in pharmacology and toxicology and is referred to as "hormesis". Hormesis can happen when an organisms biological system "overreacts" to a toxicant present at low levels. This is a well known biological reaction and does not usually indicate an environmentally adverse effect. This dose-response pattern, while non-monotonic, is still a valid dose-response relationship and point estimation techniques required by the Methods Manual should provide reliable results in most situations.

A stimulatory response is characterized by an increase in response at low concentrations. This stimulation at low concentrations can be followed by a detrimental effect at higher concentrations (see Figure 1.5.4) or by no effect at higher concentrations (see 4.). The stimulatory pattern characterized in Figure 1.5.4 is typically found with chronic, sublethal endpoints such as reproduction and growth.

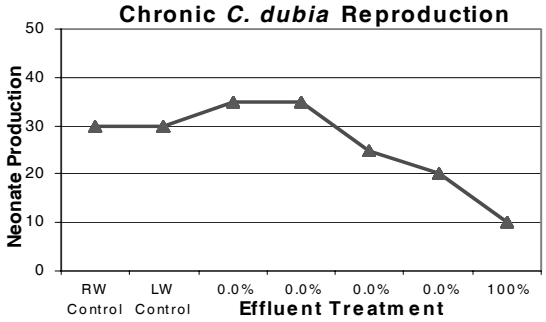

Figure 1.5.4 An example dose-response, showing stimulation at low concentrations and adverse effects at higher concentrations

4) **Stimulation at low concentrations but no significant effect at higher concentrations.** This dose-response relationship is similar to the previous example in that stimulation is observed at lower concentrations, but in this case, higher concentrations do not produce significant effects (see Figure 1.5.5). Results from point estimation techniques should be interpreted carefully when this response pattern is encountered, because statistical packages may result in endpoints that indicate toxicity at concentrations where the response is comparable to the control.

For example, the inhibition concentration (ICp) procedure used for chronic tests assumes that responses are monotonically non-increasing, meaning that the mean response for each higher concentration is less than or equal to the mean response for the previous concentration. If this is not the case, the ICp uses a "smoothing" technique that averages means (including that of the control) with those of the next highest test concentration until responses are monotonically non-increasing. In cases where the responses at the low effluent concentrations are much higher than the control, this smoothing process may result in a large upward adjustment in the control mean. This can lead to an ICp result that is less than the highest test concentration, even though this concentration was not significantly different from the control. If the response pattern shown in Figure 1.5.5 is encountered, the following should be done in addition to standard test review procedures:

a) **Evaluate the concentration range** - If the highest concentration was < 100% effluent, future tests should include higher concentrations to establish if a valid dose-response exists. This may not be necessary if the IWC is much < 100% and test results indicate no toxicity at that level and above.

b) **Evaluate control response** - It is possible that the response pattern shown in Figure 1.5.5 could result from poor control performance rather than stimulation at lower effluent concentrations. Poor control

performance could cause a toxic effect at higher effluent concentrations to go undetected. To evaluate this possibility, compare the control response to the normal control performance for the laboratory. If 1) the test exhibits a response pattern similar to that shown in Figure 1.5.5 and 2) the control response is well below the laboratory's normal range of control performance, then retesting is recommended even if the minimum test acceptability criteria have been met. For example, if a laboratory usually achieves a control mean of 25-30 neonates in the *C. dubia* chronic test, a control mean of 15-18 neonates (in conjunction with a non-ideal dose-response curve) would warrant retesting. In this situation, suppressed control performance could be considered as the cause for this response pattern rather than stimulation. A review of control performance should also investigate the possibility of poor performance in a single replicate substantially reducing the mean control response. In this case, retesting is also recommended.

c) **Evaluate the ICp calculation** - If a test exhibits the pattern shown in Figure 1.5.5 and it has been determined that this is not due to poor control performance, then discrepancies may be due to bias from the ICp smoothing technique. To determine if this is the case, calculate the % difference between the response at the IWC and the control ([*mean response at IWC/mean control response] x 100*). If the observed percent difference between the response at the IWC and the control is < 25% and the response at the IWC is not statistically significantly different from the control response, then a calculated IC_{25} of less than the IWC should be noted as anomalous and the effluent determined to be non-toxic at the IWC. If the observed difference is $\geq$ 25%, then the calculated IC_{25} should be considered valid.

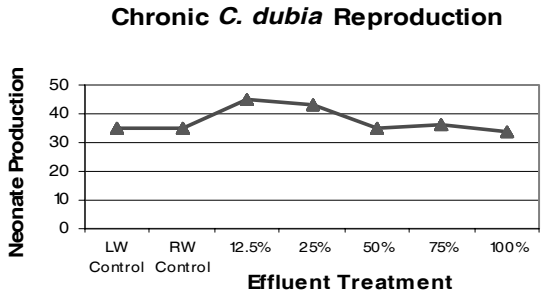

Figure 1.5.5 An example dose-response, showing a stimulatory response at low concentrations but no significant effect at higher concentrations

5) **Interrupted dose-response: significant effect bracketed by non-significant effects.** This response pattern is characterized by a single test concentration showing a significant difference from the control while adjacent higher and lower test concentrations do not differ significantly from the control (Figure 1.5.6). When this response pattern is encountered, point estimation techniques generally will yield reliable results.

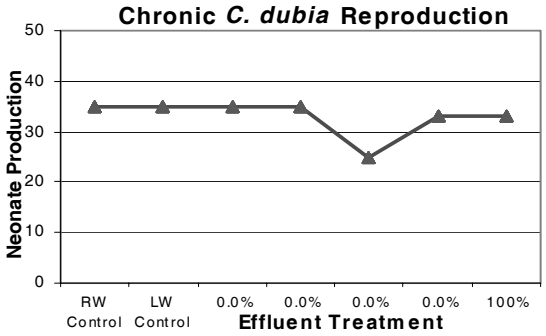

Figure 1.5.6 An example dose-response, showing an Interrupted dose-response: non-significant effects bracketed by significant effects

6) **Interrupted dose-response: non-significant effects bracketed by significant effects.** This response pattern is similar to the previous pattern in that the dose-response curve is interrupted, however, this pattern is characterized by two or more concentrations showing a significant difference from the control while an intermediate test concentration does not differ significantly from the control (Figure 1.5.7). When this response pattern is encountered, point estimation techniques will generally yield reliable results.

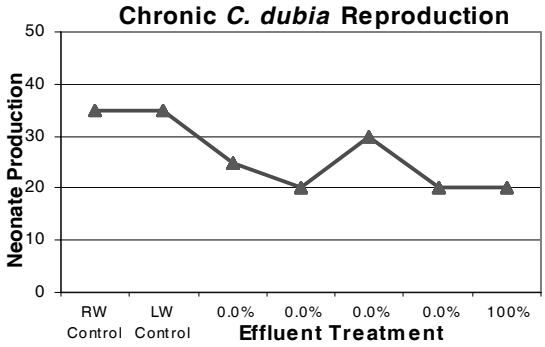

Figure 1.5.7 An example dose-response, showing an Interrupted dose-response: non-significant effects bracketed by significant effects

7) **Significant effects at all concentrations but flat dose-response.** This pattern is demonstrated in Figure 1.5.8. All test concentrations produce a response that is significantly different from the control response, but a clear dose-response relationship cannot be determined.

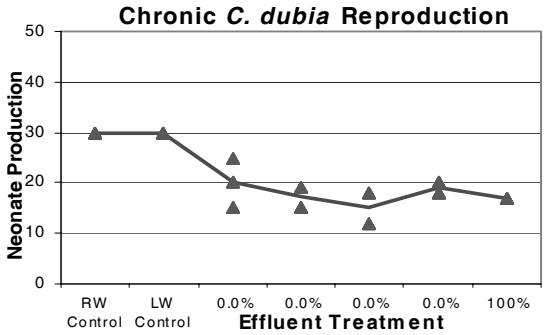

Figure 1.5.8 An example dose-response, showing significant effects at all test concentrations but flat dose-response curve

This response pattern could be due to: 1) low variability in the control, 2) an unusually high control response, 3) inappropriate dilution water or improper use of controls, (4) inappropriate test dilution series, (5) pathogen effects in the effluent, or (6) an unusual effluent-dilution water interaction. The following should be done to determine a cause for this dose-response and to determine the validity of calculated results:

a) **Evaluate the control response** - The pattern shown in Figure 1.5.8 could result from an unusually high control response. Labs are encouraged to track control performance over time. When a pattern like that in Figure 1.5.8 is exhibited, the control response should be compared to historic control performance in the lab. If the mean control response is above the normal range for that lab, a repeat test may be needed.

b) **Evaluate dilution water** - The improper use of dilution waters and controls could cause the dose-response pattern shown in Figure 1.5.8. It should be confirmed that test concentrations were compared to the dilution water control.

c) **Consider pathogen effect** - The pattern shown in Figure 1.5.8 could also be due to the presence of pathogens in the effluent. The most common identifier of pathogen effects are sporadic mortalities and high variability between replicates. The pathogen effect is more common in tests using fish species than in invertebrate testing and in chronic tests than acute tests. If within-treatment CVs for survival are >40% for effluent concentrations and relatively small for control replicates in standard synthetic water, a pathogen effect should be considered. If pathogen effects are suspected, the sample should be retested.

d) **Continued testing** - If all of the above scenarios have been investigated and have not revealed the cause of the response pattern, the results should be considered valid. However, further testing should be done in order to identify the cause of the response pattern. If an effluent consistently exhibits this response pattern, additional investigations could include chemical analysis or toxicity identification procedures.

8) **Significant effects at all concentrations with a sloped dose-response curve.** This pattern is similar to that identified in #7, except a dose-response can be identified at the higher concentrations (Figure 1.5.9). This is considered to be a valid dose-response relationship, and point estimates will generally yield reliable results.

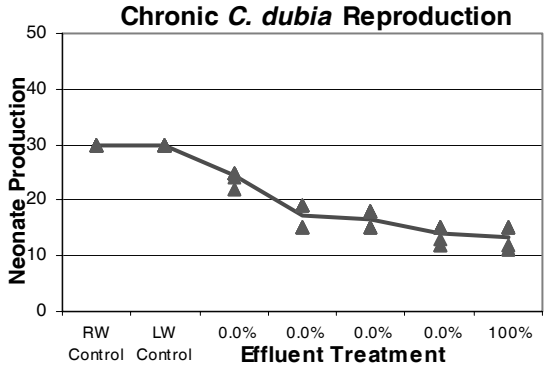

Figure 1.5.9 An example dose-response, showing significant effects at all test concentrations with a sloped dose-response curve

9) **Inverse dose-response relationship.** This pattern is characterized by a relationship in which adverse effects decrease with increasing effluent concentration (Figure 1.5.10). This is often encountered in algal growth tests and sometimes in *C. dubia* chronic tests, and is typically caused by excess nutrients in the effluent. An inverse response pattern could also be due to the presence of pathogens in the effluent. While a valid dose-response relationship is demonstrated in this circumstance, the effluent may be nontoxic since the direction of the dose-response relationship indicates decreasing adverse effects. It should be noted that while the effluent may be non-toxic, the presence of excess nutrients still may pose a potential risk to the environment due to nutrient enrichment and oxygen depletion.

An inverse dose-response pattern may also occur in tests when the dilution water used is a receiving water. In such situations, the inverse pattern can result from toxicity or a lack of necessary ions/nutrients in the dilution water. Under such circumstances, the objective of the toxicity test should be evaluated. If the objective of the test is to determine the toxicity of the effluent in the natural receiving water, then the results indicate no toxicity in the sample. If the objective of the toxicity test is to determine the absolute presence of toxicity in the effluent, the sample should be retested using a standard synthetic dilution water.

An inverse dose-response may also occur due to toxicity dependent characteristics of the effluent and dilution water. For example, if a parameter such as hardness changes with effluent concentration, and toxicity is hardness-dependent, an inverse dose-response could result (e.g., if hardness decreases with less effluent/more receiving water, and toxicity increases with decreasing hardness, an inverse dose-response like that shown in

Figure 1.5.10 would be the expected result). Since a valid dose-response is demonstrated in this circumstance, the effluent would be considered toxic and retests may be necessary.

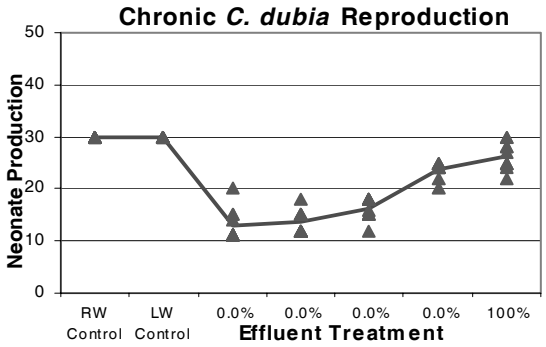

Figure 1.5.10 An example dose-response, showing an inverse dose-response relationship

Confidence Intervals

The Methods Manual requires that test endpoints be reported as an LC_{50} (acute) or IC_{25} (chronic). The 95% confidence intervals associated with these endpoints must also be reported, as an estimate of the precision (uncertainty) around the LC_{50} or IC_{25} value. The Methods Manual requires that the proper statistical method be performed using EPA or commercially available software, which generally produce a point estimate with the associated 95% confidence intervals. It is important to note that under certain circumstances confidence intervals are not produced by the software or are unreliable. This can happen if test data do not meet specific assumptions required by the statistical methods, if point estimates are outside of the test concentration range, or if specific limitations of statistical software are encountered. Confidence intervals are not used when determining compliance, but must be reported (when available) and may be used as supplemental information when interpreting test results.

The 95% confidence intervals are a measure of the uncertainty of the endpoint calculated by the statistical package. As the 95% confidence intervals of the point estimate increases (i.e., get wider), the uncertainty in that estimate of the statistical endpoint increases. Conversely, the smaller the width of the confidence intervals, the more certain one can be that the endpoint determined by the statistical program is accurate. In WET testing, confidence intervals can be a measure of intratest variability. The confidence intervals for chronic endpoints are directly influenced by the variability between replicates in each treatment and the model used to interpolate the point estimate. The confidence intervals for acute test results using a point estimate approach, however, are not influenced by variability between replicates but by the characteristics of the dose-response relationship. As discussed in Chapter 2.12 of this guidance document, the certainty in point estimates is also a function of the dilutions tested and their proximity to the actual statistical endpoint being calculated. One will get a better estimate of the LC_{50} (tighter confidence intervals) if dilutions are tested near the concentration which actually results in 50% mortality.

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